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Evaluation of *BRCA2* in the genetic susceptibility of familial esophageal cancer

Nan Hu[1], Chaoyu Wang[1], Xiao-You Han[2], Li-Ji He[3], Ze-Zhong Tang[2], Carol Giffen[4], Michael R Emmert-Buck[1], Alisa M Goldstein[1] and Philip R Taylor*[,1]

[1]*National Cancer Institute, Bethesda, MD 20892, USA;* [2]*Shanxi Cancer Hospital, Taiyuan, Shanxi 030013, People's Republic of China;* [3]*Yangcheng Cancer Hospital, Yangcheng, Shanxi 048100, People's Republic of China;* [4]*Information Management Services, Inc., Silver Spring, MD 20904, USA*

Previous studies of esophageal squamous cell carcinoma (ESCC) have shown a high frequency of allelic loss on chromosome 13q, infrequent somatic mutations in *BRCA2*, and a suggested association between a positive family history (FH+) of upper gastrointestinal cancer and germline *BRCA2* mutations. In all, 70 ESCC patients (44 FH+ and 26 FH−) were examined by direct full sequencing of germline DNA for *BRCA2* mutations. In addition, 28 family members of three of these patients and 232 unrelated healthy blood bank donor controls were examined for the mutations identified in the 70 ESCC patients. Five *BRCA2* germline mutations, including three not previously reported (N1600del, A2054P, and V2109I), were identified in six of 44 FH+ patients, but none of 26 FH− patients (14 *vs* 0%, $P = 0.078$), consistent with our previous findings (3/34 or 9% FH+ *vs* 0/22 or 0% FH−, $P = 0.27$). The cumulative frequency of *BRCA2* germline mutations in ESCC patients in this and our previous study combined is 12%, with all mutations found in FH+ as opposed to FH− cases (9/78 or 12% FH+ *vs* 0/48 or 0% FH−, $P = 0.013$). We conclude that germline mutations in *BRCA2* in ESCC patients from this high-risk area of China are more frequent in FH+ than FH− cases, suggesting that *BRCA2* may play a role in genetic susceptibility to familial ESCC.
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Introduction

Esophageal squamous cell cancer (ESCC) is one of the most common fatal tumors worldwide; however, its molecular etiology remains largely unknown. The disease occurs at a high rate in several distinct geographic regions, including Shanxi Province in northern China. Epidemiological studies have shown that tobacco and alcohol are the major risk factors for esophageal cancer (EC) in the low-risk populations of Europe and North America, but the etiology of this tumor in high-risk populations remains less clear. In addition to environmental factors (Li *et al*., 1980; Li, 1982), there are at least four lines of evidence supporting a role for genetic susceptibility in the etiology of ESCC in the high-risk populations of north central China. These include the association of a positive family history with increased risk of developing this cancer (Hu *et al*., 1991; Wang *et al*., 1993); evidence of familial aggregation of the disease (Li and He, 1986; Wu *et al*., 1989; Hu *et al*., 1992); segregation analyses among high-risk family pedigrees, which suggest an autosomal recessive Mendelian pattern of inheritance (Carter *et al*., 1992); and cytogenetic studies, which have found that chromosomal instability, as measured by increased chromosomal aberration rates, fragile sites, and/or sister chromatid exchanges, is much more common in ESCC cases and their healthy relatives than in healthy persons from families without a history of ESCC (Wu *et al*., 1989). In order to search for genes involved in the development and/or progression of ESCC in patients from Shanxi Province, previously we performed several studies of loss of heterozygosity (LOH) in ESCC patients. A genome-wide scan of allelic deletion showed a very high frequency of LOH on chromosome 13 (Hu *et al*., 2000), which we confirmed with the subsequent fine mapping of LOH on this chromosome (Li *et al*., 2001). These data also showed two common deletion regions (including one on 13q12.3–q13.1, where *BRCA2* is located) and a significantly higher frequency of LOH in patients with a positive family history (FH+) of upper gastrointestinal (UGI) cancer compared to patients with no family history (FH−). To assess whether *BRCA2* was the inactivation target for allelic loss on chromosome 13 in ESCC, we examined the entire coding sequence of *BRCA2* for mutations, using single-strand conformation polymorphism (SSCP) analysis and DNA sequencing in the same group of 56 patients. Eight mutations were identified in five patients, including three germline and two somatic mutations (Hu *et al*., 2002). Although the frequency of germline *BRCA2* mutations (9%) was relatively low, all the three

*Correspondence: PR Taylor, Cancer Prevention Studies Branch, NCI, 6116 Executive Plaza, Suite 705, Bethesda, MD 20892-8314, USA; E-mail: ptaylor@mail.nih.gov
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Table 1 Primer sequences for alterations in *BRCA2*[a]

Primer name	*Primer sequences*	*PCR product size (bp)*	*Mutation (exon)*	*Nucleotide change*
CH08 F	5′-aat tag cat gtg aga cca tt-3′	263	N1600del (11)	del 3 bp (aat)loss of one amino acid (Asn)
CH08 R	5′-caa tga ctg aat aag ggg act g-3′			
CH09F	5′-aat gtg ctt ctg ttt tat-3"	259	C315S (10)	**t**gt→**a**gtCys Ser
CH09R	5′-aca ttc atc agc gtt tgc tt-3′			
CH12F	5′-tgt gtt tat ttt gtg tag tgt-3′	268	Q2580 H (16)	ca**g**→ca**t**Gln His
CH12R	5′-gag gga ata cat aaa agt ta-3′			
BR2-E11F1	5′-acg tac tcc aga aca ttt aa-3′	200	A2054P (11)	**g**ct→**c**ctAla Pro
BR2-E11F2	5′-tag acg tag gtg aat agt g-3′			
BR2-E11R1	5′-cac tat tca cct acg tct a-3′	148	V2109I (11)	**g**tt→**a**ttVal Ile
BR2-E11R2	5′-aac cac ctt caa cat tta a-3′			
BR2-E18F	5′-tag tgc aga tac cca aaa agt-3′	230	K2729N (18)	aa**g**→aa**t**Lys Asn
BR2-E18R	5′-tta cca aga gtg caa att a-3′			

[a]Annealing temperatures were 55°C for all reactions

Table 2 Germline *BRCA2* variants identified in ESCC patients and their risk factors

No	*ID*	*Germline variant*	*Age at diagnosis*	*Sex*	*Tobacco use*	*Alcohol use*	*Pickled vegetable consumption*	*Hot food consumption*	*Family history of cancer*
1	L002-001	N1600del[a]	39	M	N	Never	Seldom	Seldom	Father (EC), maternal grandmother (EC)
2	L012-001	C315S	44	F	N	Never	Seldom	Seldom	Sister (EC), father (CC)
3	L014-001	Q2580H[a]	48	M	Y	Daily	Seldom	Daily	Paternal cousin (EC), mother (cervical cancer)
4	SHE00365	A2054 P[a]	59	M	Y	Daily	Weekly	Daily	Mother (EC), father (BC), maternal uncle (BC)
5	SHE00563	K2729N	63	M	Y	Monthly	Weekly	Seldom	Sister (EC)
6	SHE00853	V2109I	68	F	N	Never	Daily	Seldom	Brother (CC)
7	SHE00969	C315S	50	M	Y	Monthly	Never	Seldom	Mother (EC), father (CC)
8	SHE00003	K2729N	59	F	Y	Never	Seldom	Seldom	No family history of any cancer
9	SHE138	R118H	55	F	N	Never	Daily	Never	Mother (EC)
10	SHE360	C315S	55	M	N	Never	Seldom	Daily	Father (EC), brother (BC)
11	SHE437	P3300[a]	47	F	N	Never	Seldom	Seldom	Father (EC), mother (EC)

Nos. 1–8 are from the present study; 9–11 are from a previous study. EC, esophageal cancer; CC, cardia cancer; BC, body of stomach cancer; [a]not reported in the BIC as of April 2003

germline mutations detected were in patients with a family history of UGI cancer, suggesting a possible association between family history of UGI cancer and mutations in *BRCA2*.

In the present study, we selected 70 ESCC patients, including 44 FH+ (average age 53.7 years) and 26 FH− cases (average age 48.9 years), to perform *BRCA2* germline mutation testing using full sequencing (Table 1). As a part of our ongoing family study being conducted in Shanxi Province, we also had germline DNA available for evaluation from 28 first-degree relatives of three of the mutation-positive ESCC cases. Finally, we examined DNA from 232 unrelated healthy blood bank donors as controls, including 101 from Beijing and 131 from Yangcheng, Shanxi Province, to confirm that the *BRCA2* germline mutations observed were not simply novel single-nucleotide polymorphisms (SNPs) in Chinese.

Results

Full sequencing of the *BRCA2* gene revealed that six of 44 FH+ group ESCC patients (14%) had five germline mutations, while no mutations were found in the FH− group ($P = 0.078$, Fisher's exact test). The five germline mutations included four missense mutations (Q2580H, A2054P, V2109I in one patient each, and C315S in two patients) and one deletion (N1600del) (Nos. 1–8, Table 2). The potential risk factors in these patients are also shown in Table 2. No breast or ovarian cancers were reported in the families of these patients. Three of these mutations (Q2580H, A2054P, N1600del) have not been reported in the Breast Cancer Information Core (BIC) database, http://research.nhgri.nih.gov/bic. In total, 28 family members from the three FH+ patients (L002-01, L012-01, and L014-01), whose family collections were considered complete, were tested for the same mutation identified in the case from that same family. Pedigrees and genotypes for these three families are shown in Figures 1–3.

In family L002 ($N = 11$, including patient 01; Figure 1), the proband (01) was diagnosed with EC at age 39 years and determined to have a germline alteration (N1600del), which results in deletion of a single amino acid (Asn or N) in exon 11 of *BRCA2*. His father and maternal grandmother both died of EC at ages 69 and 82 years, respectively. The N1600del was identified in half of the relatives tested, including the

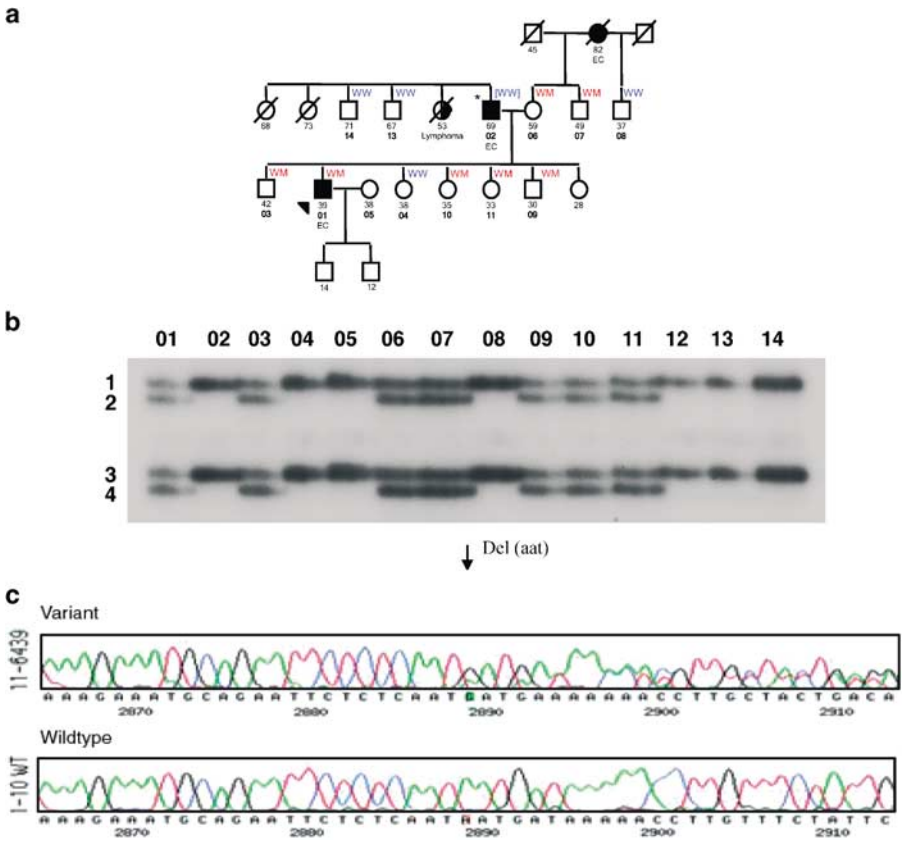

Figure 1 (**a**) Pedigree and genotypes for family L002. The arrow indicates the proband; black squares and circles indicate EC patients; WW is homozygous wildtype and WM is heterozygote for mutant allele (N1600del). (**b**) SSCP gel for N1600del in *BRCA2*. Bands 1 and 3 are the wildtype alleles, and bands 2 and 4 are the mutated alleles. (**c**) Sequences for N1600del in *BRCA2*. The wildtype sequence is -ctcaataatgat-, while the variant sequence is -ctaaatgat- (deletion of aat resulting in loss of Asp amino acid at codon 1600, as indicated by the arrow)

patient's mother, four of five siblings (03, 09, 10, 11), and one of two maternal uncles (07), but in neither of the two paternal uncles. We reconstructed the genotype for the patient's father (02) using results from his children and wife, and determined that 02 is unlikely to carry this mutation, suggesting that the N1600del was transmitted via the maternal side with segregation in the nuclear family of the proband.

In family L012 ($N=8$, including patient 01; Figure 2), a missense mutation (C315S) was identified in the proband (01), two of her siblings (03, 08), and one maternal niece (07). The proband's sister (02) also died of EC, but had no source of DNA for testing; so we reconstructed her genotype as being a mutation carrier, based on her husband (05, noncarrier), one daughter (06, noncarrier), and a second daughter (07, carrier). Taken together, these results indicate that both sisters with EC in this family carried the same mutation (C315S), although it is unclear which side of the family the mutation came from.

In family L014 ($N=12$, including patient 01; Figure 3), a missense mutation (Q2580H) was identified, including the proband (01), whose mother died of cervical cancer, and two of his siblings (05, 07). The proband's paternal cousin (02) also died of ESCC. However, 02's wife, four children, and five siblings all tested negative for this mutation; so we were unable to reconstruct 02's genotype for this alteration.

None of the five mutations found in the ESCC cases was identified in healthy individuals from either our low- or high-risk regions, with the exception of the C315S alteration that was seen in a single individual from the high-risk region. Overall, this suggests that the five germline mutations studied here are not novel SNPs in the Chinese population, as the frequencies were less than 1% for each.

Variant K2729N, identified in two of 70 ESCC patients studied here, has previously been reported as a missense mutation in the BIC database. We also found this variant in seven of 232 (3%) healthy controls (2/101 or 2% in the low- and 5/131 or 4% in the high-risk population), suggesting that K2729N is a novel polymorphism rather than a disease-specific mutation in Chinese. In addition to the alterations described above, 14 SNPs in *BRCA2* were identified in the 70 ESCC patients (Table 3). The frequencies of five of these SNPs were $>50\%$, including three nonsynonymous and two synonymous variants.

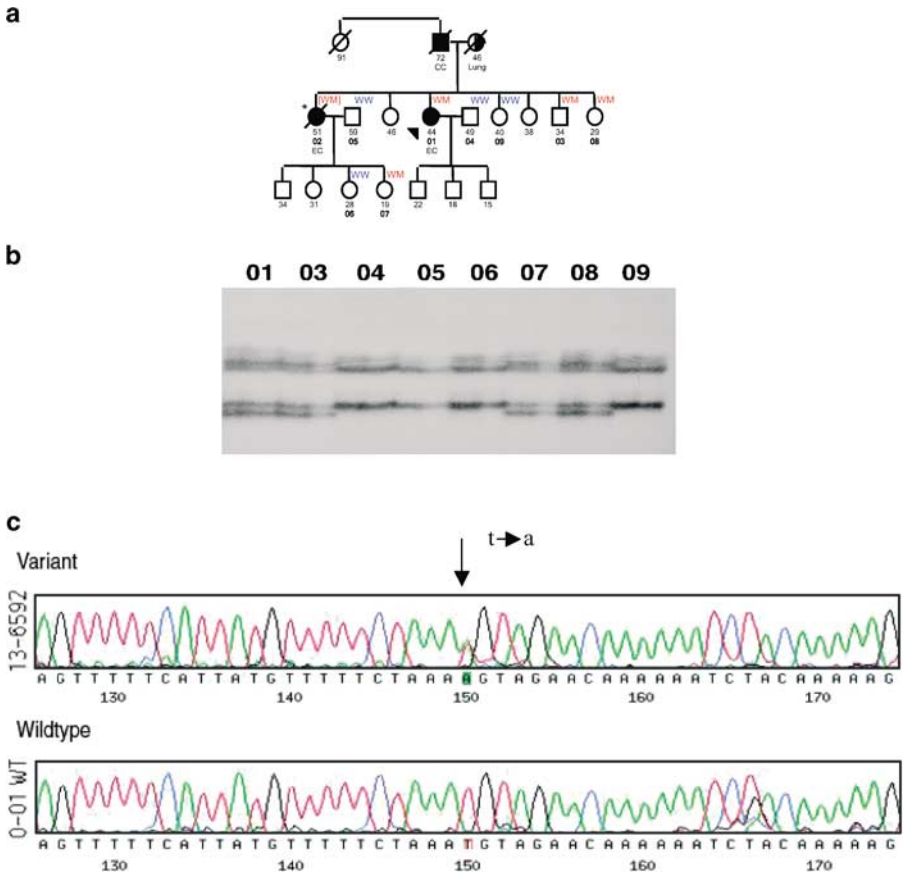

Figure 2 (**a**) Pedigree and genotypes for family L012. The arrow indicates the proband; black squares and circles indicate EC patients; WW is homozygous wildtype and WM is heterozygote for mutant allele (C315S). (**b**) SSCP gel for C315S in *BRCA2*. Bands 1 and 3 are the wildtype alleles, and bands 2 and 4 are the mutated alleles. (**c**) Sequences for C315S in *BRCA2*. The wildtype sequence is -aaa**tgt**aga-, while the variant sequence is – aaa**agt**aga. Single-nucleotide changes (t>a as indicated by the arrow) resulting in Cys to Ser at codon 315

Discussion

Germline mutations in *BRCA2* cause increased susceptibility to breast, ovarian, and other cancer types (The Breast Cancer Linkage Consortium, 1999; Nathanson *et al*., 2001), and have been identified in individuals of different races and ethnic groups with varying frequencies (Liede and Narod, 2002). Most of the deleterious alterations described in *BRCA2* are frameshift mutations that result in a truncated protein; however, in many cases of hereditary breast and ovarian cancer, amino-acid changes of unknown significance are seen. Recent studies reveal that the BRCA2 protein is required for maintenance of chromosomal stability in mammalian cells and functions in the biological response to DNA damage, as evidenced by the finding that mutations in *BRCA2* lead to chromosomal instability due to defects in the repair of double-strand and single-strand DNA breaks (Yang *et al*., 2002). Earlier studies have shown a very high level of chromosomal instability in ESCC from this high-risk area of China, observations consistent with a defect in chromosomal instability (Wu *et al*., 1989; Hu *et al*., 2000; Li *et al*., 2001). In this study, we identified a higher frequency of *BRCA2* germline mutations in FH+ ESCC patients compared to FH− patients (14% *vs* none), consistent with our previous study (9% *vs* none (Hu *et al*., 2002)). The data from these two studies combined indicate that the frequency of germline mutations is significantly higher in FH+ cases than in FH− cases (12% *vs* none; $P=0.013$; relative risk = 1.7, 95% confidence interval 1.5–2.0). Although ESCC is one of the most common fatal tumors worldwide and previous studies have shown a high frequency of LOH on 13q12 in this tumor, only two groups have reported on *BRCA2* mutations in ESCC to date, including Harada (Harada *et al*., 1999) and our group (Hu *et al*., 2002). In light of the extensive previous evidence linking ESCC with a positive family history of UGI cancer (Li and He, 1986; Wu *et al*., 1989; Hu *et al*., 1991, 1992; Carter *et al*., 1992; Wang *et al*., 1993), it is particularly interesting that we have now observed a significant difference in the rate of germline mutations in *BRCA2* between familial and sporadic ESCC patients. Taken together, these data support a role for germline mutations in *BRCA2* in the genetic susceptibility of familial ESCC in this high-risk area of China, and suggest a possible link with the high rate of genetic instability observed on chromosome 13q.

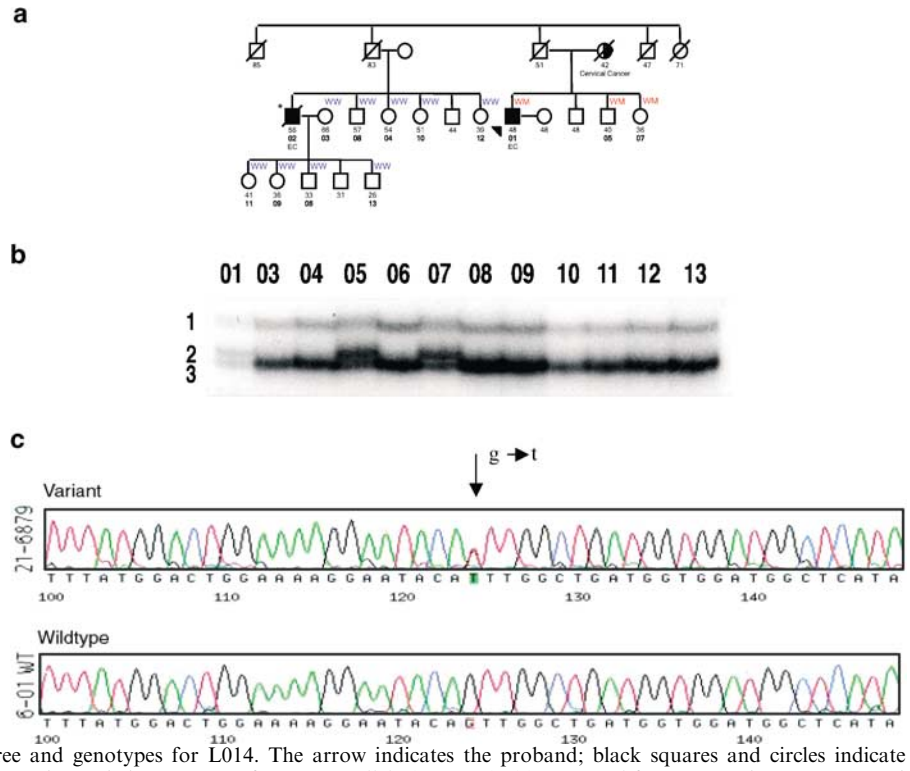

Figure 3 (**a**) Pedigree and genotypes for L014. The arrow indicates the proband; black squares and circles indicate EC patients; WW is homozygous wildtype and WM is heterozygote for mutant allele (Q2580H). (**b**) SSCP gel for Q2580H in *BRCA2*. Bands 1 and 3 are mutated alleles, and bands 2 and 4 are wildtype alleles. (**c**) Sequences for N1600del in *BRCA2*. The wildtype sequence is -ata**cag**ttg-, while the variant sequence is – ata**cat**ttg. Single-nucleotide changes (g>t as indicated by the arrow) resulting in Gln to His at codon 2580

Table 3 Summary of *BRCA2* SNPs found in 70 ESCC patients

No	*SNP name (exon)*	*# With SNP (per cent of 70 patients)*	*# With homozygous variant (%)*
1	N372H (10)	64 (91%)	38 (54%)
2	IVS16-14C>T (17)	54 (77%)	21 (30%)
3	5'UTR203G>A (2)	40 (57%)	7 (10%)
4	K1132K (11)	39 (56%)	9 (13%)
5	S2414S (14)	39 (56%)	9 (13%)
6	V1269V (11)	30 (43%)	6 (9%)
7	N289H (10)	13 (19%)	1 (1%)
8	H743H (11)	13 (19%)	0
9	N991D (11)	13 (19%)	0
10	S455S (10)	12 (17%)	1 (1%)
11	I3412V (27)	9 (13%)	0
12	IVS26-19G>A (27)	3 (4%)	0
13	K2729N (18)	2 (3%)	0
14	A2951T (22)	1 (1%)	0

Two of the *BRCA2* germline mutations identified here (C315S and N1600del) showed segregation of the mutation in the families studied, but we could not demonstrate cosegregation with disease. In one of these families (L012), both the two sibling cases carried the same C315S mutation, as did two other unaffected siblings. The C315S mutation was also observed in two other ESCC patients who were not part of the family linkage study, including one first evaluated as part of this study and another described in an earlier study (Hu *et al*., 2002). Both of these cases had a positive family history of EC (nos. 7 and 10 in Table 2). In addition to these ESCC cases with the C315S mutation, we also identified this variant in one healthy control from the high-risk area, but this person's family history of cancer is unknown. Altogether, from this and our previous study, the C315S mutation was identified in three of 78 (4%) ESCC FH+ patients, but was not seen in any of the 48 FH− patients (0%). The frequency of this variant was less than 1% in our population study ($N=411$; data not shown), suggesting that C315S is a relatively common mutation in ESCC, especially in ESCC cases who are FH+. Further follow-up of the families studied here in addition to evaluation of C315S and N1600del in additional families will be needed to determine the importance of these mutations in familial ESCC. Of note, all family members, including both carriers and noncarriers, are targeted for our ongoing follow-up program, which will continue to identify new cases and minimize misclassification by disease status in future analyses.

While segregation of disease by mutation was evident in two of our families with germline mutations, for the third family (L002), *BRCA2* mutations did not appear to segregate by disease, as one ESCC case was a noncarrier while no disease was apparent in six carriers. In this family, there is a history of EC in both sides of the family (father's side case is the father, 02; mother's side case is the maternal grandmother), which may have some implications for the unusually young age of

diagnosis for the proband in this family (i.e., age 39 years). It will be important to continue to follow this family for the development of ESCC, particularly the six carriers who currently have no clinical disease.

The role of *BRCA2* in FH− cases remains to be determined. While we have not yet seen a germline mutation in an FH− case, we have found somatic mutations in these cases, although they are relatively infrequent (2/22 or 10%), and approximately the same frequency as somatic *BRCA2* mutations in FH+ families (1/34 or 3%).

Most of the *BRCA2* germline mutations we detected in ESCC were not truncating mutations and the functional significance of these mutations is unclear. However, at least three mutations (N1600del, A2054P, and V2109I) in exon 11 are located at a conserved sequence region (BRC repeats), and these changes may influence the interaction with RAD51, a critical protein for DNA recombination and double-strand DNA break repair. Of special interest in this regard are the first four of eight BRC repeats found in the 5′ region of exon 11, which are the most conserved in mammals and bind RAD51 strongly. *BRCA2* is involved in DNA single- and double-strand repair, which suggests that genetic changes may result in chromosomal instability and increased genetic susceptibility to cancer. Although yet unproven, there are several alterations observed in this study, with some evidence suggestive of potential functional consequences, including the polymorphisms 203G>A, N372H, K2729N, and I3412V. K2729N, seen in 3% of cases and 3% of controls, is located in the conserved *BRCA2* COOH-terminal domain bound to *DSS1* (deleted in split-hand/split-foot 1 region), which can associate with *BRCA2* in the region of amino acids 2472–2957 (Marston *et al*., 1999; Yang *et al*., 2002). This variant has been reported several times in the BIC database, and a recent study reported that its frequency was less than 1% in 391 Chinese women in Tianjin (Zhi *et al*., 2002). I3412V is another interesting variant identified in 13% of ESCC patients in our study, including one patient who also had the germline mutation A2054P. This variant results in the conservative substitution of valine for isoleucine at amino-acid position 3412 in *BRCA2*. The terminal region of the BRCA2 protein, where this variant is found, can be entirely truncated. The polymorphism 203G>A was seen in 57% of the cases here and, although found in a noncoding region of *BRCA2*, a recent study showed that only chromosomes carrying G at the 203 position of the 5′UTR contributed normal mRNA, and that chromosomes carrying A (adenine) altered the reading frame and did not contribute to the production of normal BRCA2 protein (Pyne *et al*., 2000). Polymorphism N372H was identified in 91% of patients tested. Several studies have demonstrated that *BRCA2* specifically interacts with histone acetyltransferase P/CAF, which possesses histone acetyltransferase activity. This interaction requires residues 290–453 in the N-terminus of *BRCA2* where N372H is located (Fuks *et al*., 1998). It is not clear, however, whether the N372H substitution affects this binding. In the future, additional larger epidemiologic studies as well as functional laboratory studies will be required to determine the real role of these alterations in familial as well as sporadic ESCC.

In summary, we found a higher frequency of *BRCA2* germline mutations in familial ESCC patients than in sporadic patients, suggesting that germline mutations of this gene may be related to genetic susceptibility to familial ESCC in this high-risk area in China.

Materials and methods

Selection of patients and family members

This study was approved by the Institutional Review Boards of the Shanxi Cancer Hospital, the Yangcheng Cancer Hospital, and the US National Cancer Institute. After obtaining informed consent, patients were interviewed to obtain information on demographic and cancer lifestyle risk factors, including tobacco use (yes/no), frequency of alcohol use, pickled vegetable and scalding hot food consumption (never, monthly, weekly, daily), and a detailed family history of cancer (including all cancers in the first-, second-, and third-degree relatives). The definition of the family history of UGI cancer was that there were at least two UGI cancers (defined as esophageal squamous cell carcinoma, gastric cardia cancer (CC), or gastric non-CC; unlike the West, esophageal adenocarcinomas are virtually nonexistent in this area of China and are not included) in the first-, second- or third-degree relatives in a family. We selected a total of 70 patients for study, including 44 who had a history of UGI cancer (FH+ group; includes 20 from the family study and 24 from the tumor/nontumor study), and 26 with no history of UGI cancer (FH− group; all are from the tumor/nontumor study), who had a histologic diagnosis of ESCC confirmed by pathologists at the Shanxi Cancer Hospital, Yangcheng Cancer Hospital, and the NCI. None of the patients had prior therapy and Shanxi was the ancestral home for all. In the FH+ group, 20 of the 46 ESCC cases had family members aged $\geqslant$18 years, who had participated in a family study and had provided similar interview data and a blood sample for DNA testing. For three of these 20 families, data and samples were considered complete, and 28 members of these three families were evaluated as part of the current study.

Selection of healthy individuals from low- and high-risk areas

Two groups of healthy individuals were selected as controls as follows: a low-risk group included 101 individuals (males = 56, females = 45) from Beijing, where crude EC mortality rates are 18.2/100 000 in males and 8.2/100 000 in females (National Cancer Control Office, 1980); and a high-risk group was comprised of 131 healthy individuals (males = 80, females = 51) from Yangcheng county, which has one of the highest rates of EC in China (crude EC mortality rates of 167.9/100 000 in males and 94.9/100 000 in females (National Cancer Control Office, 1980)). The age range for both groups was 35–55 years.

Biologic specimen collection and processing

A volume of 10 ml of venous blood was taken from each patient prior to surgery; a similar amount was obtained from participating family members. The blood from healthy individuals was collected from blood banks in Beijing and Yangcheng. For all participants, genomic DNA was extracted and purified using standard methods.

Polymerase chain reaction (PCR) and gene sequencing

For ESCC patients, full gene sequencing of *BRCA2* in both the forward and reverse directions of approximately 10 200 base pairs, covering 26 exons, and approximately 900 adjacent base pairs in the noncoding intervening sequence, was performed by Myriad Genetic Laboratories, Inc. (Salt Lake City, UT, USA, NCI149). The specific mutations identified in patients were then examined in family members and healthy controls by PCR-SSCP and DNA sequencing, as described previously (Hu *et al*., 2001). Primer sequences for alterations in *BRCA2* are shown in Table 1.

Abbreviations

LOH, loss of heterozygosity; FH+, family history positive; FH−, family history negative; UGI, upper gastrointestinal; ESCC, esophageal squamous cell carcinoma; SSCP, single-strand conformation polymorphism; SNPs, single-nucleotide polymorphisms; PCR, polymerase chain reaction; EC, esophageal cancer; CC, cardia cancer; BC, body of stomach cancer.

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